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Ultrastructural and functional abnormalities of intestinal and renal epithelium in the SHR

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Ultrastructural and functional abnormalities of intestinal and renal epithelium in the SHR. Intestinal calcium transport, renal tubular calcium reabsorption, and plasma 1,25 $(OH)_2$ vitamin D_3 (calcitriol) levels have all been reported to be diminished in the spontaneously hypertensive rat (SHR) compared with its genetic control the Wistar Kyoto rat (WKY). In the present study, absorptive duodenal and renal tubular epithelia of 12- to 14-week-old male SHR and WKY were examined by electron microscopy to determine whether such disturbances could be related to structural abnormalities. Patchy loss of microvilli in both duodenal and proximal tubular epithelia was observed in the SHR, whereas brush border membrane was entirely normal in the WKY. Irregular spaces were observed between the basal aspects of SHR intestinal epithelial cells and their basement membrane. In addition, the average height of duodenal and renal microvilli was reduced in the SHR. Two specific markers of the brush border membrane, alkaline phosphatase and villin, as well as the cytoplasmic vitamin-D dependent calcium-binding proteins, $CaBP_{9K}$ and $CaBP_{28K}$ were determined. Duodenal alkaline phosphatase activity was reduced in the SHR, compared with the WKY: 0.145 ± 0.002 vs. 0.186 ± 0.002 IE/min · $\mu m^3 \times 10^3$ brush border, mean ± SEM, $N = 10$ pairs, $P < 0.001$. However, duodenal villin expression was not different from that of the WKY. Duodenal $CaBP_{9K}$ and renal $CaBP_{28K}$ content was diminished in the SHR: 21.0 ± 0.80 vs. 29.9 ± 2.19 μg/mg protein, $N = 6$ pairs, $P < 0.01$ for duodenum, and 4.47 ± 0.39 vs. 7.67 ± 0.54 μg/mg protein, $N = 6$ pairs, $P < 0.001$ for kidney. These data showing structural and functional abnormalities of intestinal and kidney cells in the SHR appear to reflect a disorder of transporting epithelia which may be either intrinsic or related to reduced circulating calcitriol.

Disturbances in the metabolism of calcium have been observed in arterial hypertension at both the whole organism and the cellular and subcellular levels in humans and in several experimental animal models [1, 2]. In particular, numerous abnormalities of calcium handling have been demonstrated in the Okamoto-Aoki spontaneously hypertensive rat (SHR) compared with its genetic control the Wistar-Kyoto rat (WKY). These include abnormal binding of calcium to plasma membranes [3–5], altered calcium fluxes in vascular smooth muscle [3, 6, 7], modified free cytosolic calcium concentration in platelets [8] and vascular smooth muscle [9], and altered whole-body calcium [10, 11].

Disturbances of intestinal and renal handling of calcium in the SHR have been reported by several investigators including ourselves, but the nature of the abnormality involved remains a matter of some controversy [2]. This may be due at least in part to differences in the age, gender, strain and diet of the animals as well as in laboratory techniques. Thus in the very young, 4-week-old male SHR compared with the corresponding WKY, increased intestinal absorption of calcium has been observed in association with higher levels of plasma calcitriol and increased intestinal brush border membrane fluidity [12]. In the 12- to 14-week-old SHR, on the other hand, decreased intestinal calcium absorption [13–16], reduced circulating calcitriol levels [16, 17], and secondary hyperparathyroidism [11, 18] have been reported. Similarly, hypocalciuria obtained in 11-week-old SHR [12], whereas hypercalciuria has been reported in more mature SHR [11, 19].

The question has never been addressed whether such functional abnormalities of epithelial transport in the SHR might have morphological correlates. We therefore examined intestinal and renal transporting epithelia by electron microscopy. In addition, we investigated one structural protein (villin) and three vitamin D-dependent cellular proteins, namely the brush border enzyme alkaline phosphatase, and the cytoplasmic proteins, intestinal $CaBP_{9K}$ and renal $CaBP_{28K}$ [20]. Villin, a 92 to 95 kD actin-binding protein, is found in the microvilli which compose brush borders and may be used as a marker in the study of their assembly [21]. There is no evidence of its being abnormal in rachitic chicks [22], but the situation in the SHR has not been addressed. While the content of the vitamin-D dependent calcium-binding protein (IMCAL) has been found to be decreased in the kidney and intestine of the SHR compared with that of the WKY [23], no study thus far has examined the possibility of an abnormal $CaBP_{9K}$ and $CaBP_{28K}$ content of transporting epithelia of the SHR. The vitamin D-regulated CaBP's (now also termed calbindin-D) represent an intracellu-

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lar expression of calcitriol steroid-like action. The concentration of 9 kD CaBP ($CaBP_{9K}$) in the duodenum and that of the 28 kD CaBP ($CaBP_{28K}$) in the kidney are reduced in vitamin D-deficient rats and increase after a single injection of calcitriol [20]. Although their exact role remains unknown, based upon their specific localization in the absorptive epithelium along the intestine and in the epithelium of the distal convoluted tubule in the kidney, the two CaBP's are almost certainly involved in the active calcium transport in these tissues.

Methods

Animals

Male SHR of the Wistar Okamoto-Aoki strain and normotensive WKY 12- to 14 weeks of age were used for the study of morphological changes in intestinal and renal epithelia, duodenal villin expression, duodenal and renal content of $CaBP_{9K}$ and $CaBP_{28K}$, determination of DNA and protein content, and duodenal alkaline phosphatase activity. In addition, some of these studies were repeated in 5-week old SHR and WKY. Moreover, male Wistar rats of the AF substrain were used for the measurement of duodenal alkaline phosphatase in the presence or the absence of vitamin D deficiency.

Animals were raised in a manner identical to that described previously [16]. They were obtained either at the age of four or of six weeks from IFFA CREDO, Centre de recherche et d'élevage des Oncins (L'Arbresle, France). Up to the time of receipt, the animals received diets by the supplier containing 2.0 IU/g vitamin D_3, 0.66% phosphorus and 0.75% calcium (% dry weight). From the age of receipt in our laboratory they were raised on a synthetic diet containing 0.45% sodium, 0.46% phosphorus, 1% calcium (% dry weight), and vitamin D_2, 2.2 IU/g food, until the age of either 5 weeks or 12 to 14 weeks. The animals were maintained in well lit rooms (12 hr:12 hr light/dark cycle). Studies were performed in 5-week old as well as in 12- to 14-week old SHR and WKY. Body weight (mean ± SEM) of 5-week old rats at sacrifice was 116 ± 4.8 g for SHR and 113 ± 4.3 g for WKY, and of 12- to 14-week old animals 315 ± 8.4 g for SHR and 310 ± 7.9 g for WKY.

Two additional groups of rats were raised for the determination of brush border alkaline phosphatase activity in vitamin D deficient and vitamin D replete animals. For these studies, male Wistar AF rats were obtained at the age of three weeks (weaning). One group was raised on standard laboratory chow containing 1.2% calcium, 0.95% phosphorus and 2.0 IU/g vitamin D_3 and was exposed to a 12 hr:12 hr light/dark cycle. The other was kept in the dark for 14 days with free access to water and a low calcium, vitamin D deficient diet containing 0.02% calcium and 0.3% phosphorus. At the end of this period, the animals were given daily intra-peritoneal injections of 50 μl propylene glycol containing either 10 ng (= 25 pmol) calcitriol (from Hoffmann-La Roche Laboratories, Basel, Switzerland) or ethanol vehicle for three successive days prior to sacrifice. Synthetic diets were obtained from the Centre National de Recherches Zootechniques (La Minière par Versailles, France). Animals were allowed free access to water up to time of experiments, whereas food was withdrawn at 6:00 p.m. the day before study.

Preparation of intact intestinal and renal tissue

Animals underwent pentobarbital anesthesia (40 mg/kg body wt). Duodenal segments were exposed via mid-abdominal incision, flushed via a cannula with a washing solution and then injected for *in situ* fixation with a fixative solution. The washing solution consisted of oxygenated Ringer to which 2.25 mM $CaCl_2$, 18.33 mM procain-HCl, 0.625 mM polyvinylpyrolidin (PVP, mol wt 40,000) and 50,000 IU/liter heparin were added. Osmolality was 350 mOsm/liter, pH 7.3. The fixative solution contained 3% glutaraldehyde in a 0.1 M cacodylate buffer, 4.50 mM $CaCl_2$, 2.18 mM picric acid, and 0.625 mM PVP. Total osmolality was 540 mOsm/kg, pH 7.3. Three minutes after injection, duodenal segments were excised. In separate animals, kidneys were perfused via an aortic cannula for 20 seconds and perfused-fixed for three minutes in warm solution (37°C) under a pressure of 220 mm Hg. The washing and fixative solutions were the same as above. After perfusion, both kidneys were removed.

Preparation of isolated intestinal cells

Isolated enterocytes were obtained from the duodenum by mechanical vibration for 20 minutes according to a previously described method for the study of electrolyte transport [24]. No enzymatic digestion was performed. Thereafter, they were maintained during another 20-minute period in a working buffer of the following composition (mM): NaCl, 120; Tris, 20; $MgCl_2$, 1.0; $CaCl_2$, 1.0; glucose, 10; and K_2HPO_4, 3.0; bovine serum albumin at 1 mg/ml; osmolality of buffer was 295 to 305 mOsm/kg and pH 7.4 ± 0.1. At the end of this incubation period, cells were centrifuged at 1,000 × g for 30 seconds and the pellets fixed in the above fixative solution.

Cell viability was tested using a 0.2% Trypan blue solution as described previously [25]. Four to six samples of each cell suspension taken during the 20-minute incubation period were analyzed to estimate the percent number of viable cells. More than 90% of enterocytes from both SHR and WKY excluded the dye: non-viable cells 7.73% ± 0.93 for SHR, 5.81% ± 1.29 for WKY, $N = 41$, P = NS. Using the isolated enterocyte model, we have previously reported that the sodium efflux rate constant was reduced 44% by ouabain [24], and the absence of external sodium reduced the rate of phosphate uptake by approximately 50% [26], further testifying to the viability of enterocytes under the conditions utilized in the current study.

Electron microscopy

Electron microscopy was carried out on intact duodenal mucosa ($N = 4$ pairs) sampled at approximately 4 cm distal to the pylorus for both animal strains (top and middle part of villosities in longitudinal section were embedded), and also in suspensions of isolated enterocytes ($N = 2$ pairs). For the study of renal tubular epithelium, both kidneys were examined in four rats of each strain. Intestinal and renal tissue samples were fixed with 2% glutaraldehyde, 0.1 M phosphate buffer (pH 7.4) for one hour. After washing with the same buffer, fragments were post-fixed in 1% osmium tetroxide for one hour, dehydrated in alcohol series, thereafter in propylene oxyde, and embedded in Epon.

The apical brush border structure of the absorptive intestinal epithelium and proximal renal tubular epithelium was readily

identified by electron microscopy. The abnormal aspect of the brush border and the baso-lateral side in the SHR was obvious. In order to obtain a semiquantitative analysis of the observed changes, the total surface size of cells was assessed by measuring the length of apical surface on the electron microscope screen in each section. All measurements were made using the same magnification (× 8,500). We evaluated a brush border surface corresponding to a total length of approximately 2 to 3,000 μm for each rat and established a ratio of altered to unaltered surface. Identical measurements were carried out in SHR and WKY rats. Random micrographs were taken from each section and the same measurements were performed on these micrographs. Sections from each animal were evaluated identically. In each sample of intestinal and renal tissue, eight blocks of each area (cortical and medullary regions for the kidney) were examined.

Duodenal villin expression

We have studied the villin expression on total cell extract prepared from scraped intestinal mucosa of 12-week old SHR and WKY by immunoblotting and ELISA as described previously [27]. This was carried out using monoclonal and polyclonal antibodies raised against pig villin [28].

Determination of enterocyte DNA and total protein content

Hypertrophy and increased DNA content have been reported in myocardial and renal tissue of neonatal SHR [29, 30] and in aortic smooth muscle cells of older SHR [31] in which blood pressure had been normalized. It was of importance to investigate whether comparable differences existed between SHR and WKY duodenal enterocytes. Samples for DNA determination were obtained at the end of the 20-minute incubation period. Two aliquots of 500 μl each in duplicate were obtained to estimate the mean DNA concentration in each suspension. DNA concentration was determined with calf thymus DNA as standard using the method of Burton [32]. Protein determination was carried out on the same pellet according to Gornall, Bardawill and David [33].

Duodenal $CaBP_{9K}$ and renal $CaBP_{28K}$ measurements

For the determination of CaBP content, 1 cm duodenal segments from near the pylorus region and both kidneys were rapidly removed from 5-week old and from 12- to 14-week old SHR and WKY, frozen in liquid nitrogen, and stored at −80°C. The 100,000 g supernatants were prepared according to a previously described technique [34]. CaBP was measured by radioimmunosassay as reported previously [20, 34, 35] with antibodies prepared in rabbits. The $CaBP_{28K}$ assay used antibodies to rat kidney CaBP [35] whereas the $CaBP_{9K}$ assay used antibodies raised against rat duodenal CaBP [20]. CaBP concentration has been expressed in terms of μg/mg of soluble proteins.

Intestinal alkaline phosphatase activity

The activity of duodenal brush border alkaline phosphatase was measured by an *in situ* biochemical technique using quantitative microdensitometry as described previously [36, 37]. Briefly, intestinal slices from ether anesthetized rats were incubated for 10 minutes in Tris-HCl (0.2 M) buffer at pH 8.8 at 37°C with β-glycerophosphate (20 mM) as substrate. Liberated free phosphate was precipitated in the presence of a lead reagent, and the precipitate quantitated by scanning and integrating microdensitometry. The intensity of enzymatic reaction was determined in the middle part of duodenal villi, directly over the brush border. Twenty to thirty readings were performed for each tissue sample, and a mean value calculated for each animal examined. Enzyme activities were expressed as integrated extinction (I.E.) per minute per $\mu m^3 \times 10^3$ brush border. Intestinal alkaline phosphatase determinations were carried out on duodena from 12- to 14-week old SHR and WKY, as well as from 5-week-old vitamin D-deficient animals and their controls. All of the vitamin D-deficient animals and their controls were sacrificed on a single day for estimation of duodenal alkaline phosphatase activity and plasma ionized calcium, the latter utilizing an ICA1 calcium analyzer (Radiometer, Copenhagen, Denmark). Male SHR and WKY were raised as described above and sacrificed on a single day for estimation of duodenal alkaline phosphatase activity.

Statistics

Between-group comparisons were made utilizing the Mann-Whitney U test or Student's *t*-test as appropriate. Two tailed tests were used throughout. Results are expressed throughout as means ± SEM.

Results

Electron microscopy studies in 12- to 14-week old rats

Duodenal tissue. The electron microscopic features of intact duodenal epithelium from 12- to 14-week old SHR and WKY are shown in Figure 1. Only mature enterocytes from the upper and middle part of villi were examined. Two major differences were observed in the epithelium of SHR compared with that of WKY (Fig. 1 A and B). First, a patchy loss of microvilli was apparent in all specimens from the SHR, to the same frequency in each sample, while an entirely regular brush border membrane was seen in the WKY. The surface area partially or totally lost in the SHR corresponded to approximately 10 to 15% of total microvillar surface. Most adjacent microvilli were either normal in size or larger. Some microvilli, however, appeared reduced in height in the SHR compared with the WKY, but this may have been in part due to an oblique mode of section. Altogheter, abnormal brush border was observed in most sections of intact duodenum examined and accounted thus for approximately 10 to 15% of the microvillous surface in the SHR. The appearance of the area immediately below the brush border membrane was markedly abnormal in the SHR. Its aspect was smoother than in the WKY. In the SHR, irregular, swollen spaces were often observed between epithelial cells which remained attached to each other by desmosome junctions. The basal side of the epithelial cells was also abnormal in the SHR. Unrelated to the apical changes, there were many large "bubble-like" spaces between the basal part of epithelia and the basement membrane to which they were attached (Fig. 1 C and D). The total length of basal surface area showing structural abnormalities was three-fold greater than the total length of surface of altered brush border.

Patchy loss of microvilli was also observed in enterocytes isolated from the SHR (2 separate isolation experiments, 6 blocks of several cell fragments studied from each of the 2

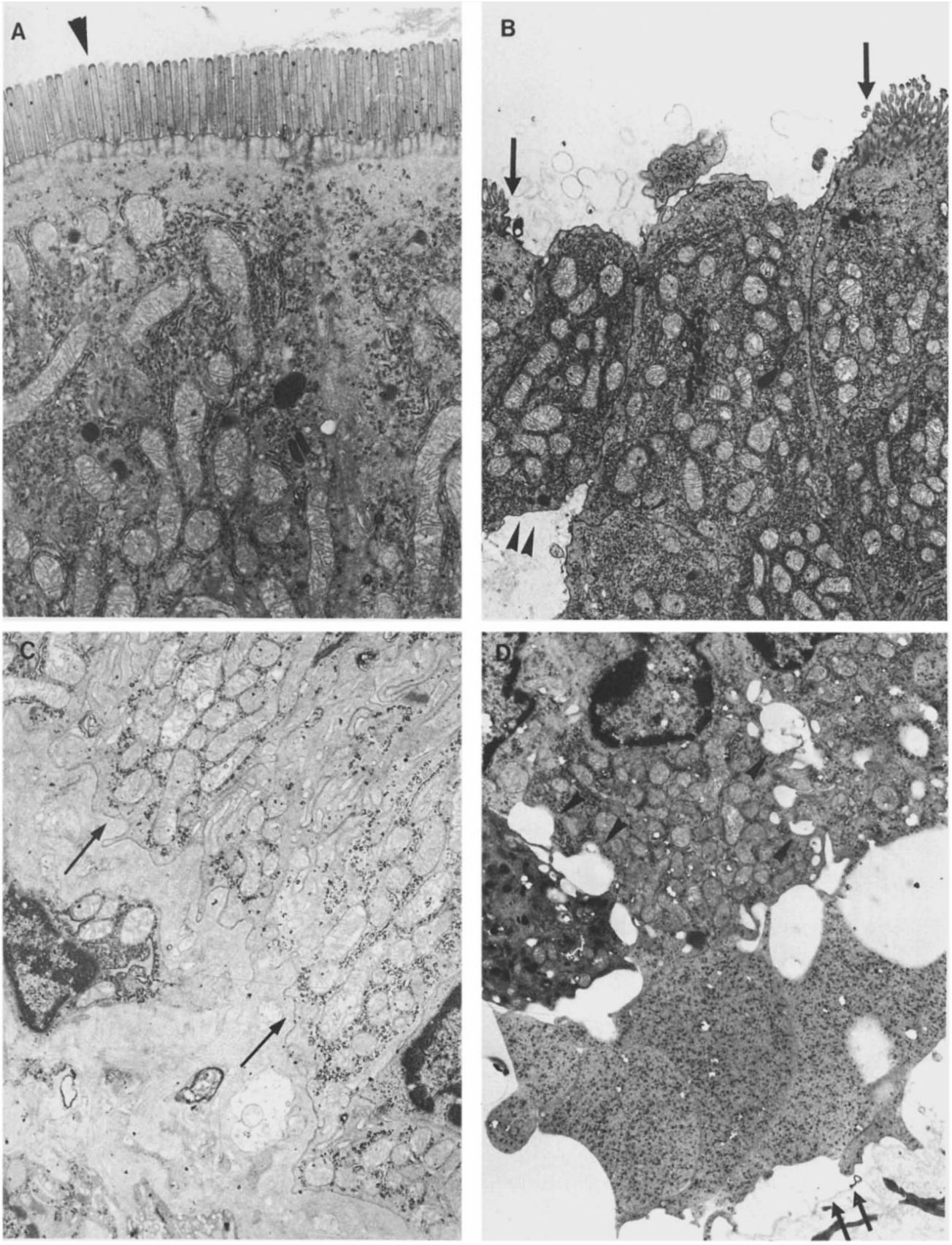

Fig. 1. *Electron micrograph of intact epithelium from mid-duodenum in 12- to 14-week old WKY and SHR.* **(A)** Aspect of normal duodenal enterocytes from the WKY. Apical zone appears with a very regular brush border (▲). Impregnation with uranyl acetate and lead citrate × 12,000. **(B)** Aspect of duodenal enterocytes from the SHR. Apical zone shows loss of surface microvilli between a normal brush border zone (→). The double arrow head indicates enlargement of later spaces (▲▲). Impregnation with uranyl acetate and lead citrate × 8,500. **(C)** Basal areas of duodenal enterocytes in the WKY showing basal zones of cells bordered by a fine basement membrane (→). Impregnation with uranyl acetate and lead citrate × 10,000. **(D)** Basal areas of duodenal enterocytes in the SHR. Epithelial cells are partially detached from basement membrane (↗↗) with bubble-like formations seen between basement membrane and bottom of cells. In addition, dilatations exist between adjacent duodenal epithelial cells (▲▲). Impregnation with uranyl acetate and lead citrate × 8,500.

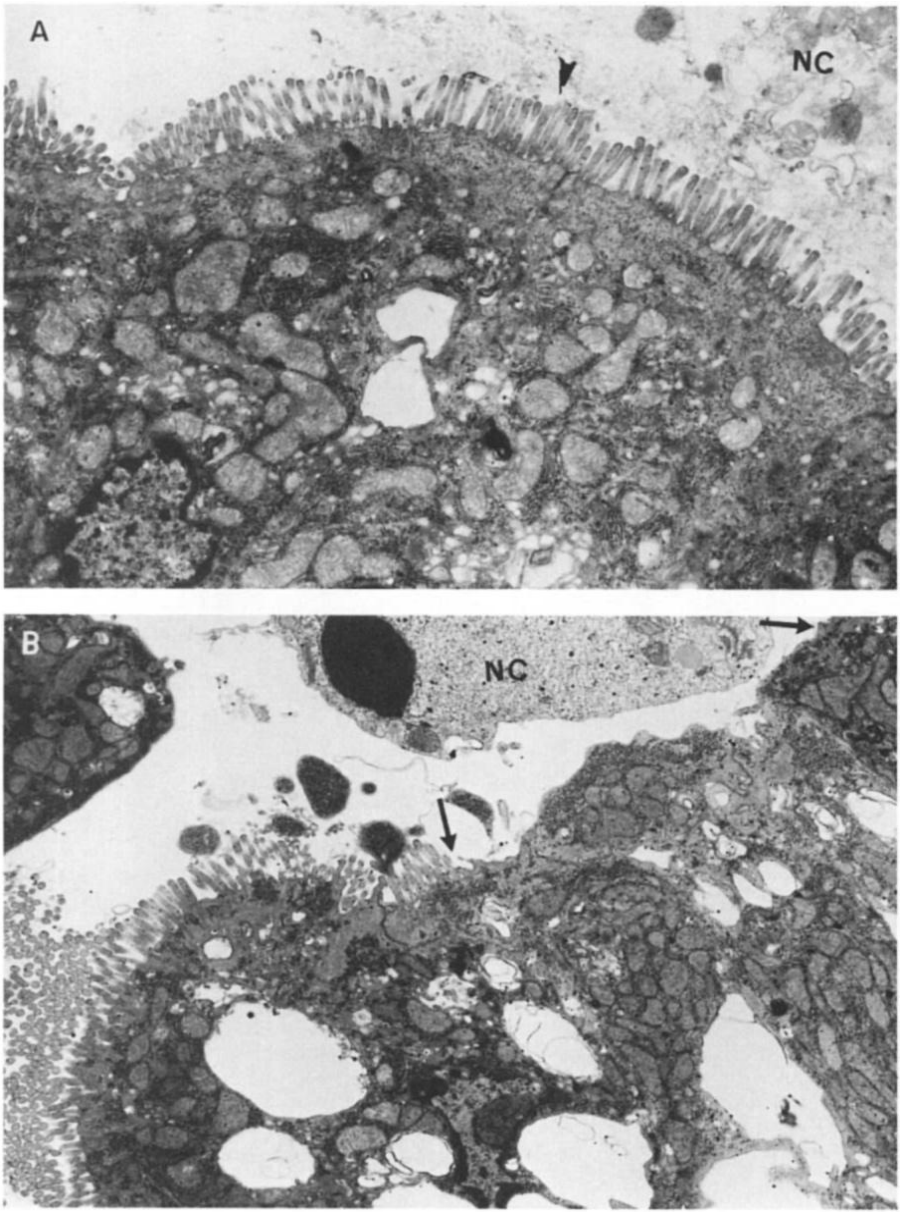

Fig. 2. *Electron micrograph of isolated duodenal enterocytes in 12- to 14-week old WKY and SHR.* **(A)** Aspect of a normal brush border (▲) in the WKY, similar to that observed in the in situ specimen. The Golgi apparatus appears somewhat dilated. Intracytoplasmic vacuoles and small dilatations between cells are observed in WKY cells. Impregnation with uranyl acetate and lead citrate × 13,000. **(B)** Electron micrograph of comparable SHR specimen. Arrows (→) indicate an area of the apical aspect of the cell lacking brush border, which is comparable to the feature shown in Figure 1. Numerous intracytoplasmic vacuoles and large intercellular dilatations are observed as in the WKY. Impregnation with uranyl acetate and lead citrate × 10,000. In specimens from both SHR and WKY, cellular debris and membrane fragments are present (NC, necrotic cellular debris).

experiments), whereas normal brush border structures were seen in all the WKY samples throughout (examination of same number of cells as for SHR; Fig. 2 A and B). Generally, three to four cells were attached together by desmosomes. These cells had the usual height of epithelial cytoplasm. Dilations between cells and vacuoles were observed in SHR and WKY cells, probably related to the technique used for cell isolation.

Renal tissue. Morphological abnormalities were observed only in the proximal renal tubule. Distal tubule, glomeruli and interstitial tissue were entirely normal. In the proximal tubular epithelium, there was a patchy loss of microvilli from the brush border membrane. In place of normal brush border, these altered apical zones appeared either as entirely smooth surfaces or, more frequently, surfaces manifesting occasional, very short microvilli (Fig. 3 A and B and Fig. 4 C). On each side of such altered zones, the remaining brush border appeared either normal or of reduced or increased height. In some cells, the area immediately adjacent to the brush border contained cytoplasmic microvesicles (Fig. 4 A). In other zones, where there was no loss of brush border structure, short and large pseudopods were observed, conceivably representing an intermediary stage between regular microvillar surface and smooth surface areas (Fig. 4 B). No morphologic changes were noted in basolateral areas of renal tubular epithelium.

Electron microscopy studies in 5-week old rats

Electron microscopic features of intact duodenal epithelium were also examined in 5-week old SHR ($N = 3$) and WKY ($N = 3$). No abnormalities of the basolateral aspect or the cytoplasm of enterocytes could be identified in the SHR at this young age,

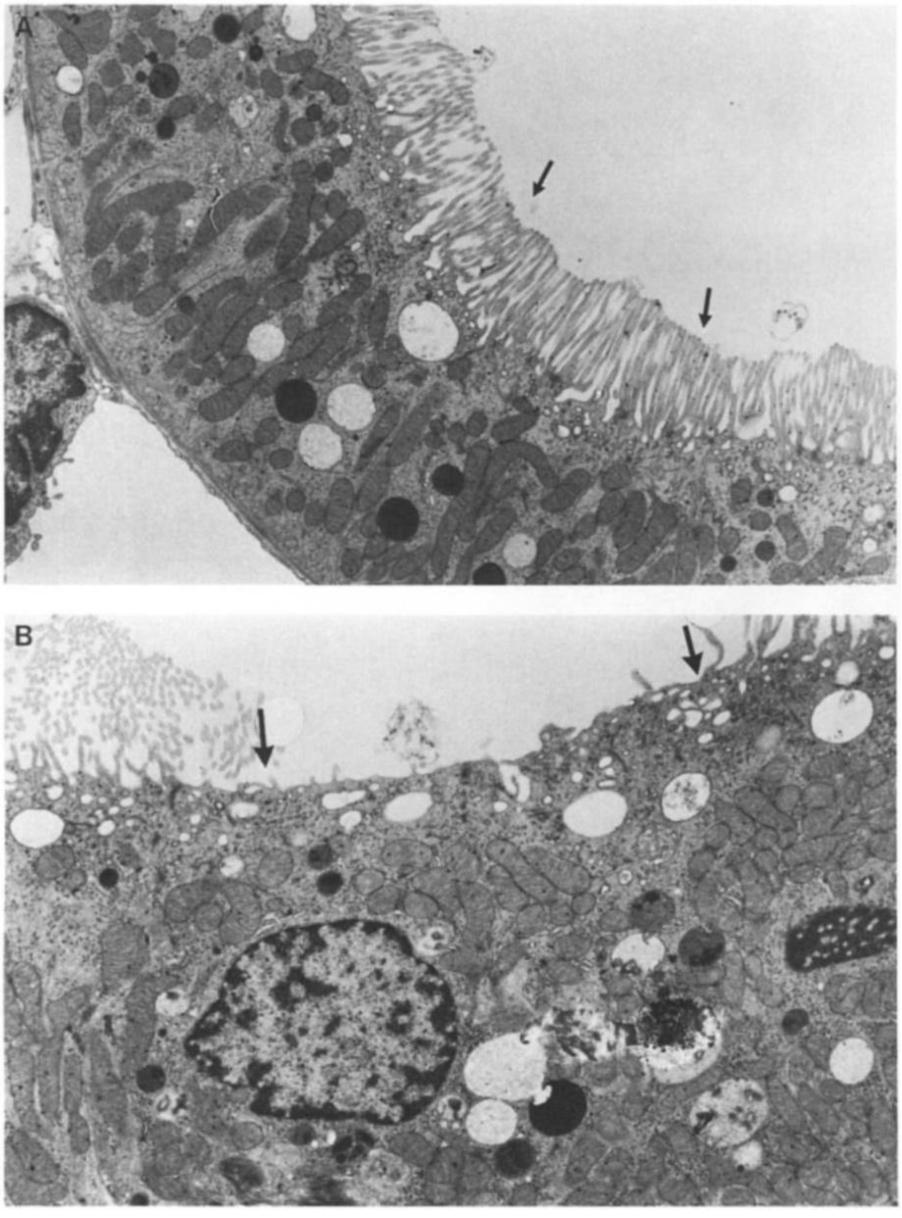

Fig. 3. *Electron micrograph of kidney proximal tubular epithelium in 12- to 14-week old WKY and SHR.* **(A)** Normal aspect of epithelium of proximal renal tubule in the WKY, with cells being regularly ranged on basement membrane (→). At the apical site, the brush border membrane has a very regular aspect (→). In the cytoplasm, cytomembranes, mitochondria and vacuoles are observed. Impregnation with uranyl acetate and lead citrate × 10,500. **(B)** Aspect of epithelium of proximal renal tubule in the SHR. Apical zone shows zones with a partial loss of brush border between areas of normally appearing microvilli (⟶). In these areas, a few clear vacuoles are also seen. Impregnation with uranyl acetate and lead citrate × 12,000.

compared with the WKY. Moreover, no areas without microvilli were observed, in contrast to the observations made in 12- to 14-week old SHR. However, in the 5-week old SHR the height of microvilli was slightly reduced in several areas. Moreover, the parallel arrangement of microvilli with respect to the underlying terminal web was not always as regular in the SHR as in the WKY.

Duodenal villin expression

No differences have been detected in villin expression on total cell extract prepared from 12- to 14-week old SHR and WKY duodenal mucosa, using either immunoblotting or the more sensitive ELISA technique (Fig. 5).

Total protein and DNA content of duodenal epithelium

Total protein, DNA, and total cell number were compared in eight SHR and seven WKY at the age of 12 to 14 weeks. No significant difference was found in protein, DNA, protein/DNA ratio or number of cells obtained between both strains of rats (SHR vs. WKY: protein, 3.27 ± 0.38 vs. 3.22 ± 0.22 mg/ml; DNA, 8.69 ± 1.34 vs. 7.43 ± 0.93 μg/ml; protein/DNA, 0.40 ± 0.03 vs. 0.46 ± 0.04 mg protein/μg DNA; total number of cells obtained, 1.90 ± 0.16 vs. 2.01 ± 0.13 × 10^6 per duodenal segment, P = NS for all comparisons.

Thus the present study of total protein and DNA content of duodenal enterocytes revealed no differences between the SHR and the WKY, suggesting no major changes in cell size or nuclear ploidy in the duodenum of the SHR.

Duodenal alkaline phosphatase

Figure 6 shows that in normal Wistar AF rats made vitamin D deficient, duodenal alkaline phosphatase activity was decreased compared with vitamin D deficient rats that had received a daily dose of 10 ng calcitriol for three days (+ vehicle vs. + calcitriol:

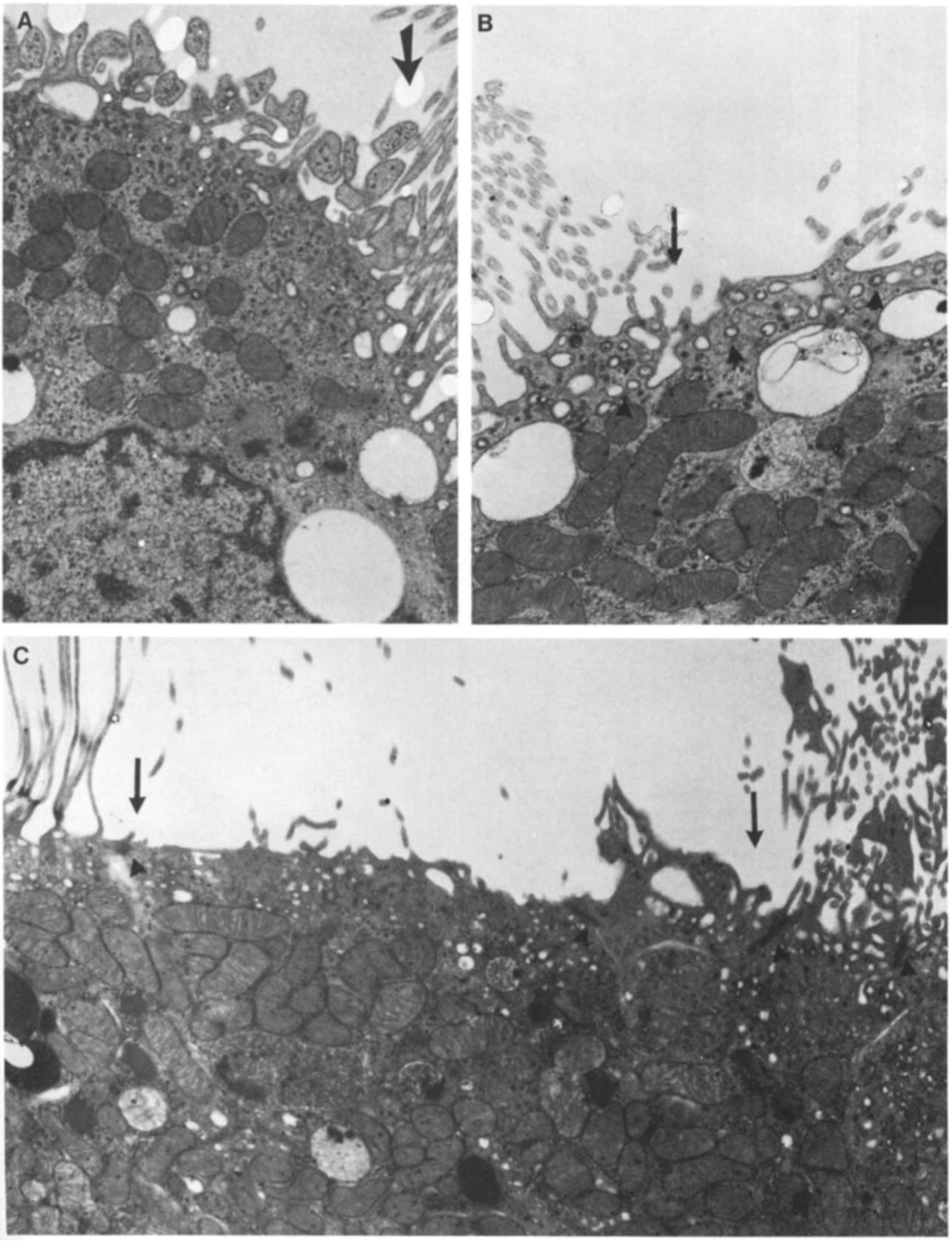

Fig. 4. *Electron micrograph of kidney proximal tubular epithelium in 12- to 14-week old WKY and SHR.* **(A)** Peculiar feature of the apical zone of proximal tubular epithelium in the SHR. Between areas of normal brush border (→) short, markedly swollen microvilli are observed, conceivably representing the first stage of changes at the level of brush border or underlying cytoplasm. Impregnation with uranyl acetate and lead citrate × 12,000. **(B)** Another feature of altered apical zone of proximal tubular epithelium in the SHR, with loss of microvilli and presence of many small vesicles (▲▲). Nearly normal brush border membrane (→). Impregnation with uranyl acetate and lead citrate × 12,000. **(C)** Large area of altered apical zone of proximal tubular epithelium in the SHR (within arrows), adjacent to microvilli which appear increased in size. Based on the presence of desmosomes, three epithelial cells appear to be altered in this tubule section. Impregnation with uranyl acetate and lead citrate × 12,000.

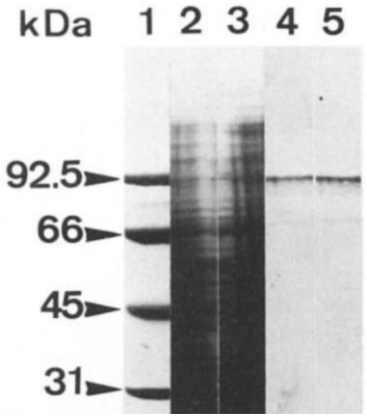

Fig. 5. *Duodenal enterocyte villin expression.* Villin expression in 12- to 14-week old SHR and WKY. Lane 1: Molecular weight markers in kDaltons. Lanes 2 and 3: SDS-PAGE, total cell extracts from WKY (lane 2) and SHR (lane 3), visualized by Coomassie blue staining. Lanes 4 and 5: Nitrocellulose replica of same SDS-PAGE with total cell extract from WKY (lane 4) and SHR (lane 5) probed with rabbit antivillin serum. Visualization of Ag-Ab complexes using anti rabbit IgG.

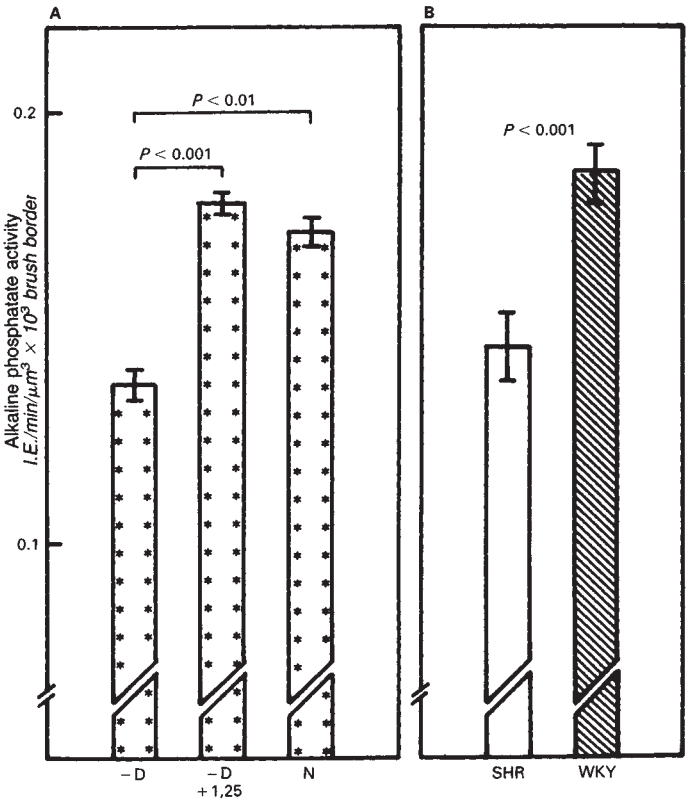

Fig. 6. *Alkaline phosphatase activity (by microdensitometry) in the brush border membrane of the enterocyte.* Alkaline phosphatase as integrated extinction per min per $\mu m^3 \times 10^3$ brush border. **(A)** Three days prior administration of 10 ng daily calcitriol to vitamin D deprived Wistar AF rats led to an increase in duodenal alkaline phosphatase activity to level found in normal rats of the same age. **(B)** Duodenal alkaline phosphatase activity was reduced in SHR compared with WKY, $N = 10$ pairs. Symbols are: Wistar (⊡); WKY (▨); SHR (□).

0.137 ± 0.003 vs. 0.179 ± 0.002 IE/min · $\mu m^3 \times 10^3$ brush border, $P < 0.001$, $N = 10$ pairs. Vitamin D-deficient animals ($N = 10$) also had significantly lower levels of activity than normally raised vitamin D-replete animals ($N = 3$); deficient vs. normal: 0.137 ± 0.003 vs. 0.172 ± 0.003 IE/min · $\mu m^3 \times 10^3$ brush border; $P < 0.01$; Fig. 6). Plasma ionized calcium increased significantly in the animals treated with calcitriol, from 0.74 ± 0.02 nmol/liter before calcitriol to 0.83 ± 0.02 mmol/liter at sacrifice, $P < 0.01$, but was unchanged in those administered vehicle alone (0.72 ± 0.01 mmol/liter before vs. 0.76 ± 0.02 mmol/liter after; P = NS). Figure 6 indicates that duodenal alkaline phosphatase activity was significantly reduced in 12- to 14-week old SHR compared with the WKY (0.145 ± 0.002 vs. 0.186 ± 0.002 IE/min · $\mu m^3 \times 10^3$ brush border; $P < 0.001$; $N = 10$ pairs).

Intestinal and renal CaBP

Figure 7 shows that in the SHR, decreased contents of epithelial $CaBP_{9K}$ were found in the duodenum, proximal and distal jejunum, and the ileum, compared with the WKY at both the age of 5 weeks and of 12 to 14 weeks. Similarly, the $CaBP_{28K}$ content of proximal renal tubular epithelium was lower in the 12- to 14-week old SHR ($N = 6$) compared with that of the WKY control ($N = 6$) of same age: 4.47 ± 0.39 versus 7.67 ± 0.54 μg/mg protein, $P < 0.001$.

Discussion

In the present electron microscopy study, we have demonstrated for the first time the presence of structural abnormalities in transporting epithelia of the SHR. The most striking abnormality seen in preparations of both isolated cells and whole duodenum and of intact proximal renal tubule epithelium in the 12- to 14-week old SHR was a patchy loss of normal brush border membrane. In addition, there were changes in the basal aspect of cells in intact duodenum. In the 5-week old SHR, only minor changes of the enterocyte brush border membrane were seen. No such abnormalities were observed in any of the tissue specimens obtained from control WKY rats at either age.

It is of note that there is increasing evidence of membrane abnormalities in a variety of cells in hypertension [38, 39]. The present abnormalities are unlikely to be artifacts induced by fixation technique since no such abnormalities were observed in the WKY rat whose tissues were prepared at the same time and in an identical manner. It is therefore highly probable that the obvious differences observed between 12- to 14-week old SHR and WKY rats at the brush border and the basolateral membrane site of transporting epithelia represent structural differences with likely functional consequences.

Changes in brush border membrane structure have also been observed in vitamin D deficiency. Thus, Sampson and Krawitt [40] carried out electron microscopy on the duodenum of vitamin D-deficient rats and reported reduced height and diameter of the microvilli, but no disorganization akin to that demonstrated in Figures 1, 2 and 3. In the ileum of vitamin D deficient chicks, Wong et al [41] found the same minor abnormalities of the microvillus as Samson and Krawitt but in addition, after incubating the ileum for 80 minutes with the polyene antibiotic filipin, observed widespread changes similar to those seen in the epithelia of the SHR in the present study.

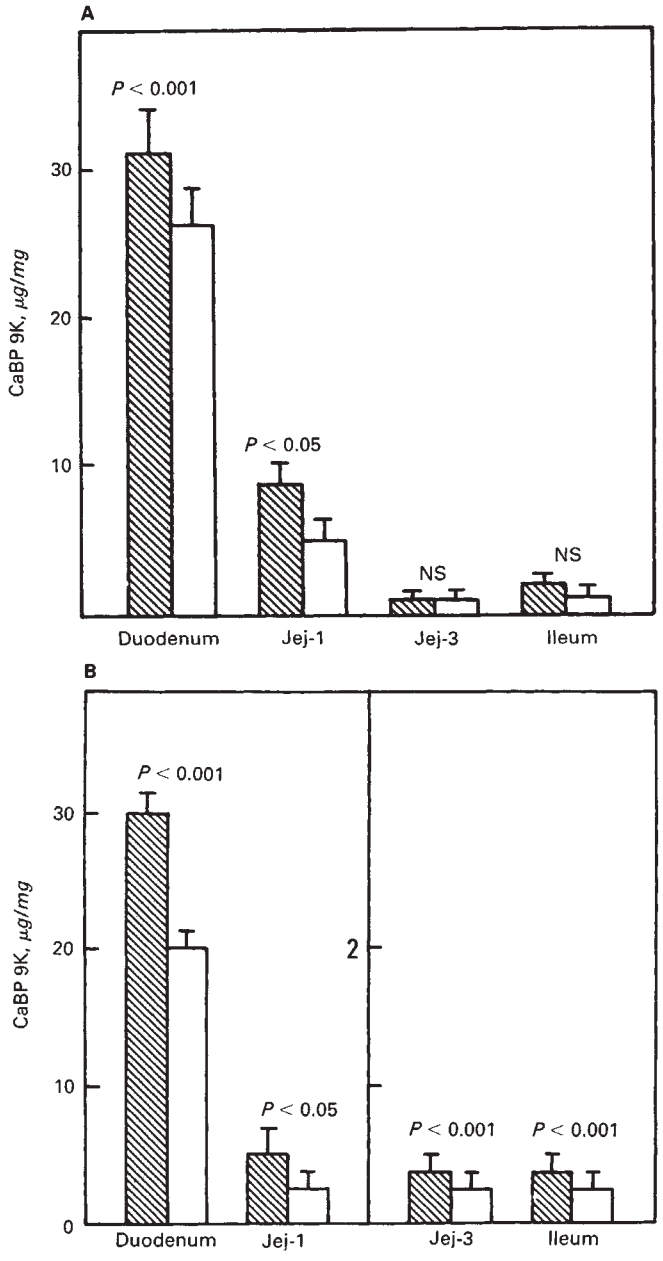

Fig. 7. *Small intestinal CaBP content.* Epithelial $CaBP_{9K}$ concentrations have been determined in 4 segments of the small intestine of SHR (□) and WKY (▨). The four segments studied were duodenum, first and third part of jejunum, and terminal part of ileum. **(A)** 5-week old rats; **(B)** 12- to 14-week old rats. Each column represents mean ± SEM of 6 rats.

Therefore, it seems unlikely that the abnormal appearance of the SHR's duodenal brush border membrane results solely from reduced circulating calcitriol, as shown in our previous study [16]. It has recently been demonstrated that intraluminal calcium may influence the physical state and lipid composition of rat enterocyte plasma membranes in vivo [42]. In view of the evidence of abnormal subcellular calcium handling in the SHR [2], it is conceivable that the concentration of calcium in the brush border microenvironment of SHR intestine differs from normal and that this might give rise to the abnormalities demonstrated.

The fact that only 10 to 15% of SHR enterocytes were modified by electron microscopy would suggest, but does not prove, that only part of total brush border membrane protein is modified or deficient. Our attempt to detect possible changes of proteins involved in the structure of the brush border membrane was unsuccessful. Thus, the amount of villin (a major structural protein of the microvillar skeleton with a high affinity for calcium) that was found in the duodenal epithelium of the 12- to 14-week old male SHR was similar to that found in its control, the WKY. However, the detection of a small decrease of intestinal villin content might have been missed by our technique of determination. Alternatively, villin could be present in normal amounts in the SHR but abnormally assembled within the cell. It is of note that the interaction between microvillar actin and villin is calcium-dependent, and that unphysiologically high calcium concentrations at the site of the microvillus core filament lead to collapse of the ordered actin-villin bundle [43]. Calmodulin, which is also abundantly present in the microvillus core filaments, appears to exert a protective role against high local calcium concentrations and their deleterious effect on microvillus structural integrity. It is possible that in the SHR, there are alterations in the interaction between actin, villin, calmodulin and calcium which are as yet unelucidated. It has been shown, however, that the dependence on Ca^{2+} of the three major, high-affinity Ca^{2+}-binding proteins in the enterocyte is hierarchically decreasing in the following order: CaBP > calmodulin > villin [44]. Therefore, the protective effect of CaBP and calmodulin in preventing marked increases of local Ca^{2+} concentrations would normally argue against the possibility that very high concentrations of the cation build up at the level of the cytoskeleton, allowing an activation of villin's severing and perhaps barbed end-capping activity [45].

In contrast to our negative finding with respect to quantitative changes of the structural brush border protein villin in the SHR, we found evidence of abnormal function of an other brush border protein, namely alkaline phosphatase, as demonstrated by the present microdensitometry study. Our observation of reduced duodenal alkaline phosphatase activity in the SHR confirms the recent findings of Gilles-Baillien, Carlier and Rorive [46], who used different methods. There is much evidence for the dependence of intestinal alkaline phosphatase activity on vitamin D status [47] and we have confirmed this in the present study. A dose of calcitriol close to the physiological intake [48] administered to vitamin D deficient rats had the effect of normalizing previously-considerably reduced duodenal alkaline phosphatase activities. It is noteworthy that alkaline phosphatase activity found in the SHR's duodenum was intermediate between that of vitamin D deficient rats and that of vitamin D replete rats and the WKY (Fig. 6). While it is possible that lower duodenal alkaline phosphatase activity in the SHR reflects decreased circulating calcitriol levels, it may also result from the intrinsically abnormal brush border membrane demonstrated by electron microscopy. In addition to vitamin D and other factors, shifts in calcium movements per se have also been shown to modify intestinal alkaline phosphatase activity [49], and this could be an alternative explanation for the changes observed in the SHR.

Finally, we observed a decrease of intestinal $CaBP_{9K}$ content and renal proximal tubule $CaBP_{28K}$ content in the 5-week as well as in the 12- to 14-week old SHR. These proteins are almost certainly intracellular peptide carriers involved in calcium translocation across intestinal and renal epithelia, mainly directed from the mucosal to the serosal side. The novel finding in our study of such a decrease of CaBP content along the whole small intestine and also in the kidney is in accord with the diminished active calcium transport previously reported in these tissues for the adolescent and mature male SHR [11, 13–16, 19]. This decrease may be related to the concomitant reduction in circulating calcitriol concentration in the 12- to 14-week-old male SHR [13, 15], in keeping with the notion that calcitriol is the major regulator modulating the gene expression of CaBP synthesis [50], even though CaBP gene regulation may also be influenced by the ambient calcium concentration itself [51]. It is of note that recently the SHR has also been shown to have a decreased concentration of IMCAL [23], another vitamin D-dependent protein with high calcium affinity which is located in the brush border membrane and which may also play a role in the translocation of the cation across the plasma membrane of the enterocyte. Our finding of a reduced activity of alkaline phosphatase in the brush border of the SHR is consistent with the latter observation. Finally, the possible functional role of reduced plasma calcitriol levels is also suggested by our previous finding that the administration of exogenous calcitriol normalized the decrease in active intestinal transport of calcium in the SHR [16].

The cause and the mechanisms underlying the structural and functional abnormalities observed in transporting epithelia in the SHR are unclear. Complex epithelium-matrix-mesenchyme interactions govern the maturation of transporting epithelia [21, 52]. It is possible that in the SHR, the matrix proteins required to induce normal epithelial maturation are abnormal. Abnormal vitamin D metabolism may play a role in the development of abnormal structure and function of intestinal and renal epithelia of the mature SHR. Alternatively, a primary disturbance of cellular calcium handling may be the fundamental cause of the abnormalities we have observed, as also recently suggested by Schedl, Wilson and Horst [53].

In conclusion, we have investigated the electron microscopic appearance of enterocytes and proximal renal epithelium as well as epithelial proteins including alkaline phosphatase, $CaBP_{9K}$, $CaBP_{28K}$, and villin. We found evidence of abnormal structure and function, mainly at the site of the brush border membrane. The possible connection between such abnormalities of transporting epithelia and hypertension is unclear and is worthy of further study.

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